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MONITORING RELATIONSHIPS BETWEEN CORTICOSTERONE AND SNAKE FUNGAL DISEASE IN TIMBER RATTLESNAKES (CROTALUS HORRIDUS) IN WESTERN KENTUCKY

John Bromley Hewlett

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Signature page

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ABSTRACT

Over the past four decades, emergent fungal diseases have been the most devastating relative to species declines and extinctions. While most research has focused on fungal diseases affecting amphibians and bats, less has focused on diseases like snake fungal disease (SFD), caused by *Ophidiomyces ophiodiicola* (*Oo*). SFD was first described in 2006 in North America within a Timber rattlesnake (*Crotalus horridus*) population in New Hampshire. Since then SFD has been documented in 19 US states, one US territory (Puerto Rico), and Europe. SFD causes high mortality in some species, including the Eastern Massasauga rattlesnakes (*Sistrurus catenatus*), which is an endangered species. Given the ecosystem services snakes provide (e.g., control of rodent vectors) and known linkages between disease and stress, it is important to understand the relationships between SFD and stress in snakes as well as the distribution of the disease.

One indicator of stress is circulating corticosterone (CORT) concentrations. Along with epinephrine, CORT helps mediate the fight-or-flight response by mobilizing energy stores and modulating the immune system. Previous research has evaluated relationships between baseline corticosterone (CORT) levels and SFD status; however, to my knowledge no research has investigated relationships between SFD and elevated CORT, CORT reactivity (percent increase from baseline to elevated CORT), or CORT variability. My first objective was to examine Timber rattlesnakes for the presence or absence of SFD and measure their circulating CORT levels in one-month intervals. I conducted a three-year study evaluating relationships between SFD disease state and CORT, including baseline and elevated CORT and CORT reactivity along with individual variability of each of these metrics. From May through September 2018 and 2019, I captured and surgically implanted radio-transmitters into 20 snakes. Following

implantation, I tracked snake movements at least once per week and obtained blood and swab samples once per month during the active season (May - September) from 2018 - 2020. I found no difference in baseline and elevated CORT between SFD positive and negative individuals except for August, when elevated CORT was 2 times greater in SFD positive snakes. I also found a positive correlation between the proportion of times a snake tested positive for SFD and variability in CORT reactivity. Greater CORT levels in August could have implications for SFD positive snakes as they prepare for the winter inactive period by way of suppressed immune function. Moreover, snakes that test positive for SFD more often show highly variable, inconsistent CORT measures.

To target conservation efforts, we must also delineate the geographic scope of SFD and obtain a better understanding of host taxa. For objective 2, I conducted a descriptive survey to monitor the presence of SFD in snakes in Western Kentucky. I focused my survey efforts in the Jackson Purchase region, including the Land Between the Lakes National Recreation Area, where data on SFD is largely lacking. I collected 124 individual snakes across 18 species. Sixteen percent of the snakes sampled tested positive for SFD with the majority being terrestrial species, particularly the Crotalines. This may indicate taxa specific differences in infection risk. My data on the linkages between SFD and CORT and my monitoring efforts may help managers implement and better strategize conservation and management practices for snakes in an increasingly human dominated landscape.

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CHAPTER 1

INTRODUCTION

In the past three decades the number of epizootic and zoonotic infectious diseases has increased due to landscape degradation and biodiversity loss associated with the Anthropocene (Daszak et al., 2000). The emergence of infectious diseases has had effects on non-human animal populations (e.g., human outbreaks of Ebola virus disease correlate with Lowland Gorilla (*Gorilla gorilla*) outbreaks, resulting in population declines (Bermejo et al., 2006) and affected entire communities (e.g., Passerine diversity is negatively correlated with West Nile virus; Keesing et al. 2010) (Blehert et al., 2009; Lips et al., 2006). Disease emergence can influence humans directly (e.g., illness or death) or indirectly, through the loss of ecosystem services (e.g., control of mosquito populations, freshwater filtration, pollination of crops and native plants) related to biodiversity and the 'dilution effect' whereby it is posited that there is an inverse relationship between species diversity and infection prevalence (Civitello et al., 2015; Khalil et al., 2016). Consequently, we must better understand the implications of both epizootic and zoonotic diseases on wildlife populations for conservation and human health.

Among emerging diseases, fungal diseases are poorly understood (Ghosh et al., 2018) and are most severe in terms of effects on animal populations (Scheele et al., 2019). Specifically, Chytridiomycosis and White-nosed Syndrome (WNS) have resulted in declines and local extinctions of amphibian and bat populations, respectively (Fisher et al., 2012). Similarly, snake fungal disease (hereafter SFD), is caused by *Ophidiomyces ophiodiicola (Oo)*, which is a saprophytic fungus that can be free living in the soil (Stengel et al., 2019). Snakes affected by SFD are characterized by skin lesions and behavioral changes (Lorch et al., 2016). The lesions generally appear as dry yellow crusts that occur on the facial, dorsal, and ventral regions, and can

present as nodules and ulcerations with some of the nodules being subdermal (Lorch et al., 2016). Fungal hyphae can also penetrate compromised areas (e.g., injuries) of the stratum corneum layer of the epidermis with granuloma formation (Lorch et al., 2015). The disease can also result in flaking of the skin in a process known as dysecdysis (Franklinos et al., 2017). Lesions associated with SFD may result in negative fitness consequences for infected individuals by interfering with their ability to feed (Lorch et al., 2015) causing snakes to exhibit abnormal basking behaviors (Clark et al., 2011; McBride et al., 2015; Snyder et al., 2020), which may result in an increased risk of predation, and/or affecting osmotic balance and subsequently leading to water loss in individuals with clinical symptoms (Agugliaro et al., 2020). Though less severe cases may be sloughed during molt, infection occasionally leads to mortality (Lorch et al., 2015).

Though there is uncertainty regarding the origin of SFD, SFD has become widespread in snake populations across eastern North America and in some cases has been linked to population level declines (Allender et al., 2015). Given risks to snake populations that are already experiencing other stressors (e.g., habitat loss, climate change, pollution), understanding the influence of SFD on snake populations is crucial. Moreover, SFD is a concern because snakes provide valuable ecosystem services. For instance, snakes serve as both predators and prey of many species across diverse taxa and may help regulate populations of animals considered pests (Hisaw and Gloyd, 1926). Snakes are important for seed dispersal (Reiserer et al., 2018), and theoretical models suggest that some snake species may help mitigate the transmission of tick-borne diseases (e.g., Lyme Disease) (Kabay et al., 2013).

Diseases such as SFD may be associated with stress. Physiological or psychological stress (i.e., anything that results in an alteration to homeostasis [Nelson and Kriegsfeld, 2018])

contributes to infections via immunosuppressive mechanisms (Dohms and Metz, 1991; Råberg et al., 1998). Stressors may involve a myriad of stimuli such as extreme temperatures, lack of access to food or water, and/or anthropogenic factors including landscape fragmentation and conversion and climate change (Gabor et al., 2018; Nelson and Kriegsfeld, 2018; Refsnider et al., 2015). Stress leads to the secretion of multiple hormones, including glucocorticoids such as corticosterone (hereafter CORT) (Nelson and Kriegsfeld, 2018), from the adrenal cortex as part of the "fight-or-flight response". The secretion of these hormones can have a wide array of effects as glucocorticoid receptors can be found on virtually all cell types (Papadimitriou and Priftis, 2009). The secretion of the hormones in response to a stressor is regulated at the level of the brain by the hypothalamic-pituitary-adrenal (HPA) axis (Bellavance and Rivest, 2014; Nelson and Kriegsfeld, 2018; Smith and Vale, 2006) and the HPA axis is critical in regulating physiological systems during stress by increasing blood glucose levels and modulating metabolism (Stephens and Wand, 2012). Cardiovascular and immune functions are also affected by HPA activity (Bailey et al., 2003; Burford et al., 2017; Stephens and Wand, 2012), making the system important with respect to disease susceptibility and or the ability to recover from disease (Stefferl et al., 1999).

The stress response can be adaptive or maladaptive depending on environmental circumstances (McEwen, 2007). Maladaptive stress responses could include exaggerated or blunted (i.e., a failure to adequately mount a response) response to external stimuli. In mammals, both exaggerated and blunted stress responses are associated with negative health outcomes due to loss of homeostatic regulation (Brotman et al., 2007; Lovallo, 2011; Phillips et al., 2013). In vertebrates other than mammals, such as reptiles, anthropogenic factors (e.g., roadway construction) have been associated with blunted stress responses (Owen et al., 2014).

Elevated baseline plasma CORT correlates with disease susceptibility and resistance (Dunlap and Schall, 1995; Gross and Colmano, 1971; Stewart et al., 1988). Baseline CORT is defined as the level of CORT found in an unstressed state (Romero and Reed, 2005). Up regulation of the HPA-axis occurs in amphibians infected with *Batrachochytrium dendrobatidis* (*Bd*) the causative agent of Chytridiomycosis (Gabor et al., 2013) and may play a role in WNS infection in bats as well as SFD infection in pygmy rattlesnakes (Bouma et al., 2010; Lind et al., 2017; Reeder and Kramer, 2005; Reeder et al., 2004).

In addition to understanding relationships between stress and SFD, we also need to better understand the geographic distribution of SFD. The effects of SFD may vary within or between species across geographic ranges leading to implications for conservation, management, and epidemiology. While disease surveillance has been conducted in the state of Kentucky, most of the surveys were in the bluegrass and central regions. Much remains unclear regarding the presence or absence of SFD in the extreme western end of the commonwealth where several ecosystem types including bald cypress wetlands occur. These ecosystem types are not found elsewhere in the state. The Jackson Purchase (8 counties west of the Tennessee River) of Kentucky represents a unique ecoregion characterized by upland oak-hickory forests, extensive agriculture, and wetlands such as bald-cypress sloughs. Furthermore, there may be different climatic and weather patterns in the Jackson Purchase because of the unique confluence of 4 major river systems that may be different in the rest of the commonwealth. In addition to Kentucky, surveys have been conducted in the surrounding states of Tennessee and Virginia with *Oo* being ubiquitous on the landscape (Guthrie et al., 2016; Snyder et al., 2020).

Studies have demonstrated snakes with SFD tend to have greater baseline CORT; however, there has been no research to date in squamates investigating the association between elevated

CORT or CORT reactivity (the percent increase, or magnitude of change from baseline to elevated CORT) (Wingfield et al., 1992) and SFD. While baseline CORT can be informative, understanding the relationship between these CORT variables and disease is needed to fully assess the effects of stress on wild populations. Moreover, while some surveys on SFD have been conducted in Kentucky, there is a lack of data from western Kentucky. Thus, the objectives of my study were to:

Objective 1. Investigate the relationship between SFD *and* 4 metrics of stress: baseline CORT, elevated CORT, CORT reactivity, and CORT variability.

Objective 2: Assess the presence of SFD across snake species in Kentucky west of the Cumberland River.

CHAPTER 2

TIMBER RATTLESNAKES WITH SNAKE FUNGAL DISEASE EXHIBIT GREATER VARIABILITY IN CORTICOSTERONE REACTIVITY

Synopsis

In mammals and birds, it is well established there is a clear relationship between baseline circulating glucocorticoid hormones and infectious (i.e., viruses) and non-communicable (i.e., cardiovascular, metabolic) diseases. While prior research has suggested there is an association between baseline CORT and snake fungal disease (SFD), no study has examined relationships between disease state and measures of elevated CORT, CORT reactivity, and CORT variability. In this study I evaluated relationships between SFD and baseline CORT, elevated CORT, CORT reactivity, and CORT variability in Timber rattlesnakes (*Crotalus horridus*). I used radiotelemetry to track two cohorts (n = 10 per cohort) of Timber rattlesnakes (n = 20) weekly during the active period from May to August 2018 and 2019 [cohort 1] or 2019 and 2020 [cohort 2]. I obtained baseline and elevated blood samples along with tissue swabs from each snake once per month. Baseline CORT is the ambient CORT concentration in an unstressed state, and elevated CORT is the concentration following exposure to a stressor. Baseline CORT and CORT reactivity did not differ between SFD positive and SFD negative individuals within any month. Elevated CORT was 2 times greater in SFD positive snakes compared to SFD negative snakes in August but did not differ in other months. Additionally, I found a positive relationship between variability in a snake's CORT reactivity and the proportion of times a snake tested positive for SFD. One explanation for elevated CORT in August may involve a compounding effect between disease and stress associated with movement back to hibernacula in preparation for hibernation. A more variable response among recurrently sick individuals likely involves dysregulation of the

HPA-axis because of compounding stress over time and could potentially carry fitness consequences.

Background

Reptiles are in decline globally because of habitat loss and destruction, overharvest, pollution, and climate change (Gibbons et al., 2000). Though reptile species are declining generally, we have relatively less information on squamates (e.g., snakes and lizards) as compared with other non-squamate reptiles, such as turtles. Of specific concern are snakes because they are often vilified, difficult to study because of their cryptic nature, and they provide important ecosystem services as predators and prey (Kabay et al., 2013). The potential downstream consequences of snake population declines could be substantial to ecosystems and the other animals, including humans, that rely on them.

Snake fungal disease is an emergent infectious disease caused by *Ophidiomyces ophiodiicola* that was first documented in North America in 2006. In 2006, dermal lesions were observed on the bodies of Timber rattlesnakes in New Hampshire and these lesions were confirmed to be associated with a fungal pathogen (Clark et al., 2011). Since 2006, SFD has been documented across the eastern United States and in Europe (Franklinos et al., 2017). Researchers have since discovered SFD in at least one endangered species, the Eastern Massasauga (*Sistrurus catenatus*), and in this population SFD was often lethal (Allender et al., 2015). Other studies suggest the effects of SFD on snake populations are variable and endpoints of the disease (i.e., mortality versus recovery) may differ based on stressors experienced by populations, including temperature and other seasonal variables (Lind et al., 2018). Though data on SFD is being generated rapidly, much remains unknown with regard to disease dynamics in wild populations.

One area where data are lacking involves a broader understanding of stress and its implications for the ecology and epidemiology of SFD. Current research has focused on measurement of baseline CORT (Lind et al., 2017, 2010, 2018), a primary hormone involved in an organism's stress response (Nelson and Kriegsfeld, 2018). Baseline CORT is the concentration prior to exposure to a stressor, whereas elevated CORT is the hormone concentration following exposure to a stressor. Pygmy rattlesnakes (*Sistrurus miliarius*) in central Florida showing severe SFD symptoms had greater baseline CORT than individuals who were mild/moderately infected or otherwise healthy (Lind et al., 2018). To my knowledge no research has investigated relationships between infection with SFD and elevated CORT (levels of CORT experienced in response to a stressor), CORT reactivity (defined as the percent increase from baseline to elevated CORT; Wingfield et al., 1992), or CORT variability.

Many studies have documented that there are deleterious effects on health associated with elevated levels of baseline CORT (Lovallo, 2011; Phillips et al., 2013). Evidence also suggests there are negative health consequences (e.g. cardiovascular disease, increased infection risk) associated with either an exaggerated or blunted stress response (Lovallo, 2011; Phillips et al., 2013; Waller et al., 2016). Diseases like SFD could lead to dysregulation of the HPA-axis, which can result from chronic or repeated stress and could lead to maladaptive reactions to stressors (e.g., habitat loss/fragmentation, pollution, climate change). Although variability in baseline and elevated CORT levels and CORT reactivity over time could indicate dysregulation of the HPA-axis, little has been done to understand variability in CORT titers in wildlife as it relates to disease because of the difficulty and costs associated with acquiring long-term data sets.

Research has documented relationships between baseline CORT and SFD (Lind et al., 2018) but no research has been conducted on relationships between SFD infection and elevated

CORT, CORT reactivity, or the variability of each of these metrics (hereafter CORT variability) in either wild or captive populations. I evaluated the relationship between SFD status and baseline CORT, elevated CORT, CORT reactivity, and CORT variability in a population of Timber rattlesnakes. I implanted transmitters and tracked 20 timber rattlesnakes and monitored them monthly during their active season (May to September) for circulating plasma CORT concentrations (baseline and elevated) and assessed their SFD disease status between 2018 and 2020. I hypothesized there would be a relationship between SFD infection and baseline CORT, elevated CORT, CORT reactivity and CORT variability. I predicted that snakes infected with SFD would have greater levels of baseline and elevated CORT, CORT reactivity, and CORT variability. Broadening our understanding of relationships between CORT and SFD infection will allow for a greater insight into the effects anthropogenic stress may have on wild populations.

Materials and Methods

Study Site

I conducted my research on the Kentucky section of the Land Between the Lakes National Recreation Area (LBL) (Figure 2-1) situated between the Tennessee and Cumberland Rivers. Land Between the Lakes consists of 69,201 ha of upland oak-hickory hardwoods interspersed with prairie and agriculture. LBL is currently managed by the U.S. Department of Agriculture Forest Service. All research was conducted with a USDA Forest Service special use permit (permit #LBL18178) and a state educational collection permit issued by the Kentucky Department of Fish and Wildlife Resources (permit #SC1811061). The protocols I followed were approved by the Murray State University IACUC.

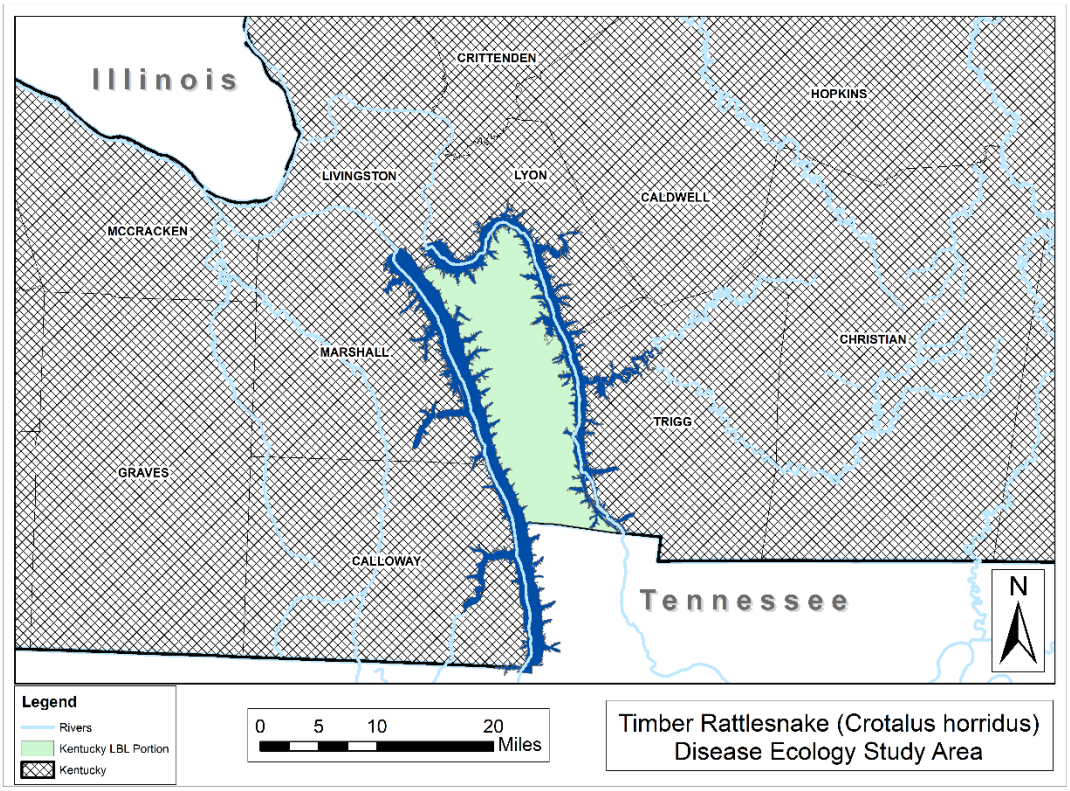

Figure 2-1. A map of the Kentucky portion of the Land Between the Lakes National Recreation Area where 20 Timber rattlesnakes were captured, transmittered, and tracked between May 2018 and October 2020.

Study Species and Collection

I conducted my study on timber rattlesnakes, which are a species of conservation concern in North America because populations are declining across their range (LaGrange et al., 2014; MacGowan et al., 2017; McBride et al., 2015; Rudolph and Burgdorf, 1997). The timber rattlesnake's life history is characterized by late maturation and low reproductive output, and when coupled with increasing anthropogenic pressure could place the species at greater peril.

I collected 20 timber rattlesnakes from May 1st to September 6th in 2018, and 2019 (n = 10 snakes per year). Roads were driven between sunset and 0200 h. I stopped collecting snakes during the first week of September to allow for adequate healing from surgeries prior to hibernation.

Surgical procedure

Radio transmitters (Advanced Telemetry Systems, Model R1535, 15 g) were surgically implanted intracoelomically in 20 timber rattlesnakes (16 M and 4 F) following Reinert and Cundall (1982) with minor modifications. Animals were brought into the lab in a 5 gallon bucket the night before the procedure. Prior to all surgeries, a Virkon™ solution was used to sterilize all surgical instruments, including the transmitter, and the surgical area. During the surgical procedure, everyone present utilized sterile procedures, including masking and the use of sterile gloves by the person performing the surgery. To prepare for the surgery, the snakes were safely removed from the bucket their head secured within a plastic tube. Once in the tube, I administered an anesthetic (approximately 5 mL of vaporized isoflurane) immediately via manual pumping of a 500 ml polypropylene bottle with a plastic tube attached to the end of the bottle and snake tube. Anesthesia was achieved in approximately 30-45 minutes and verified by the loss of skeletal muscle tone and ability to see the heart beating through the ventral wall. An "X" was placed over the heart using a permanent marker so I could monitor the heart rate during the procedure.

During anesthesia, I used three rayon swabs wetted with 80 µl of deionized water to swab the dorsal, ventral, and facial areas of each snake. I passed the swabs over the length of the snake 5 times on both the dorsal and ventral sides and swabbed the loreal pit and facial area through the end of the end of the tube. If clinical lesions were present, additional swabs were used to sample

individual lesions and the lesions were photographed. Once swabs were taken, I broke off the tips into a 1.5 mL microcentrifuge tube and stored them at -20˚ C.

Following swabbing, I recorded the total length (defined as the length from the tip of the snout to the base of the rattle) and the snout-to-vent (SVL) length (defined as the length from the snout to the opening of cloaca) of each animal. Once the snake was fully anesthetized, I rolled the snake on to its lateral aspect with the ventral region facing away and the surgical area was re-sterilized with a 7.5% povidone-iodine antiseptic scrub. An incision was made with a scalpel 2 scale rows from the ventral surface at a position 75% of SVL. I inserted blunt hemostats into the incision to divide tissues, followed by using my finger to divide tissues further. I identified and breached the coelomic membrane and irrigated the incision with physiological saline. All anatomical landmarks were identified, and the transmitter was gently inserted into the coelomic cavity with the whip antenna directed to the anterior end of the individual. A small incision was made, and a tomcat catheter was threaded under the skin as a guidewire for placing the whip antenna subcutaneously. The incision was then flushed with a solution of 2% chlorhexidine. I used vicryl absorbable sutures to close the primary incision and stopped anesthesia via the removal of the vaporizing tube from the end of the snake tube. The animal was fully resuscitated and placed in a sterile holding container on a thermostatically controlled heating pad until release within 24 h of capture.

Field data collection

I located each rattlesnake using radiotelemetry at least once per week from March to October. Occasionally, I would lose a snake’s signal and conduct aerial telemetry using a Cessna 172 aircraft. A standard yagi antenna was mounted to the exterior wing strut on the pilot side of the aircraft and the coaxial cable threaded through a partially opened pilot side window. The

receiver was then connected to the intercom system of the aircraft and tuned to the appropriate frequency. An additional individual occupied the co-pilot seat of the aircraft with a Garmin GPS in hand to record data points when passing overhead. I later located the snake via homing to confirm its location. Locations obtained aerially were generally < 200 m from the snake's true location.

Monthly from May to September (2018) and May to August (2019 and 2020), I collected two plasma samples from each snake during homing between 0700 and 1200 h. Once the snake was observed visually, we started a stopwatch to measure the time to blood draw and considered a blood draw under 5 minutes representative of baseline CORT levels (see data analysis and results section). When the animal was safely tubed, a syringe was flushed with a ~ 0.1 mL 150 u/mg heparin solution in deionized water until the walls of the syringe were coated with heparin sodium. Then a 21 5/8-gauge needle was inserted into the caudal vessels posterior to the cloaca. The needle was inserted until it hit the spine and was slightly withdrawn before the plunger was pulled. Approximately 0.5 mL of blood was extracted from each snake. The cap, including the hypodermic needle, was removed from the syringe prior to discharging the blood into a 1 mL microcentrifuge tube to reduce hemolyzation of the sample. Once the blood was placed into the microcentrifuge tube it was placed on ice in the field and samples were later stored at -20˚ C. The snake was then placed in a 5-gallon bucket with a screw on lid and I repeated the process 60 minutes later to collect the elevated CORT sample. Although most snakes were available for monthly sampling, two females were gravid for an entire season and one female occupied the vicinity of a high voltage power substation for two months and no blood or swab were samples collected during these times.

Glucocorticoid Assays

Within 4 h of collection in the field, microcentrifuge tubes were spun for 10 minutes at 2200 rpm and the plasma was collected. For quantifying CORT concentrations, I used the Arbor Assay™ Detect X Enzyme Immunoassay kit for Corticosterone. I added 5 μL of sample plasma into individual vials followed by the addition of 5 μL of steroid dissociation reagent to each sample vial. I then vortexed all vials briefly and allowed the mixture to sit for 5 minutes at room temperature. I added 490 μL of assay buffer to each vial to achieve a 1:100 dilution and made standard solutions following the assay protocol. Following the addition of the assay buffer, I vortexed each sample briefly and then I pipetted 50 μL of plasma samples and standards into the assay plate wells. I added 25 μL of both DetectX® Corticosterone Conjugate and DetectX® Corticosterone Antibody to sample and standard wells, less the antibody was not added to the non-specific binding (NSB) wells, and after an hour rinsed the plate four times with wash buffer. After rinsing, I added 100 μL of TMB substrate to each reaction well and waited an additional 30 minutes before adding 50 μL of Stop Solution to each well. I used an 800TS Microplate reader to read the plate at 450 nm and Gen5 software to analyze the data.

DNA Extraction procedure

Prior to DNA extraction, I cleaned the bench and pipettes with DNAoff and rinsed with 100% ethanol. I put approximately 100 mg of 0.5 mm zirconium beads into O-ring tubes (with conical bottom) containing the sample swab. Prepman Ultra lysis buffer (125 μl) was added to tubes to cover the beads and swab. I placed sample tubes in a disruptor and homogenized them for 45 seconds and centrifuged the samples for 30 seconds at 13,000 x g to settle all material to the bottom of the tube. I repeated both processes in stepwise fashion and placed the tubes on a heat

block at 100° C for 10 minutes. Tubes were then removed and allowed to cool for an additional 2 minutes. I then centrifuged the samples at 13,000 x g for 3 minutes and used 200 μL filtered pipet tips to extract the supernatant from each sample. Finally, I combined the supernatant for all samples from a specific time spot and placed each in a separate 1 mL microcentrifuge tube and labeled and stored at -20° C.

qPCR procedure

The presence of *Ophidiomyces* genes were evaluated using quantitative polymerase chain reaction (qPCR). The target is the Internal Transcribed Spacer region of the ribosomal DNA complex based on a real-time PCR protocol developed by Bohuski et al. (2015). This region has been characterized as an identifying region of the ribosomal DNA complex for fungi (Bohuski et al., 2015). Fungi represent the second largest kingdom of eukaryotes following the animals (Blackwell, 2011), but limitations have existed in species identification (Schoch et al., 2012). The ITS region is used in DNA barcoding for fungal identification instead of the more typical mitochondrial cytochrome *c* oxidase subunit 1 gene found in animals due to its (cytochrome C) composition and variability (Schoch et al., 2012). For my qPCR, I utilized a QuantiFast Probe PCR +ROX Vial Kit along with Bioresearch Technologies targeted forward and reverse primers and probes. The ABI 7500 Fast PCR Machine was used to read reaction well content in the Applied Biosystems® MicroAmp® Fast Optical 96-Well Reaction Plate. I classified snakes as SFD positive if they had lesions and a positive skin swab (Baker et al., 2019). If individuals had internalized disease, that condition could not be evaluated for this study.

Data Analysis

I used program R (R Core Team, 2021) and the packages ‘lme4’ and ‘lmertest’ (Bates et al., 2015; Kuznetsova et al., 2017) to run generalized linear models (GLMs) to assess the influence of mass, length, and sex on the log of elevated and baseline CORT and CORT reactivity (i.e. the magnitude of change between elevated and baseline CORT (Wingfield et al., 1992) and the effects of time to first blood draw on the log of baseline CORT. As none of these variables were significant, I did not include them in further models and pooled data from males and females for analyses. I ran GLMs to assess the influence of SFD status (+ or -) on the log of baseline and elevated CORT and CORT reactivity each month. I also used GLMs to assess the relationship between the proportion of times an individual snake tested positive for SFD and the average and coefficient of variation (CV) of baseline CORT, elevated CORT, and CORT reactivity. These data were calculated only using male snakes that I was able to sample between 4 or 5 times (all but one individual was sampled 5 times) over the same months. For models, I considered independent variables with $P < 0.05$ and 95% CIs of the beta coefficients that did not cross 0 significant.

Results

Seventy-five percent (n = 15) of snakes tested positive for SFD at least once during my study and 60% of individuals that tested positive for SFD had clinically significant lesions. The proportion of individuals testing positive was highest in May and lowest in August (Figure 2-2). Overall mortality was 20% (n=4), with two of these mortalities testing positive for SFD at the time of death. One emaciated individual had been sick (diarrhea indicative of internal parasites) at the time of transmitter implantation and died of exposure in February of 2019. Another

individual died from suspected depredation. I could not attribute either of the 2 SFD positive deaths primarily to SFD as opposed to some other cause.

Mass, length, sex, and time to first blood draw (baseline only) had no influence on baseline or elevated CORT or CORT reactivity (Table 2-1). I found no difference in baseline CORT between SFD positive and negative snakes across all months (Figure 2-3.) Elevated CORT did not differ in most months, but in August elevated CORT was 2 times greater in SFD positive snakes as compared with SFD negative snakes (Figure 2-4.) I found no difference in CORT reactivity between positive and negative snakes across all months (Figure 2-5). I also found no relationship between the average or CV of baseline CORT or elevated CORT and the number of times a snake tested positive for SFD (Figure 2-6, Table 2-3). Average CORT reactivity also did not differ, but there was a positive correlation between the number of times a snake tested positive for SFD and the CV of CORT reactivity (Figure 2-6, Table 2-3).

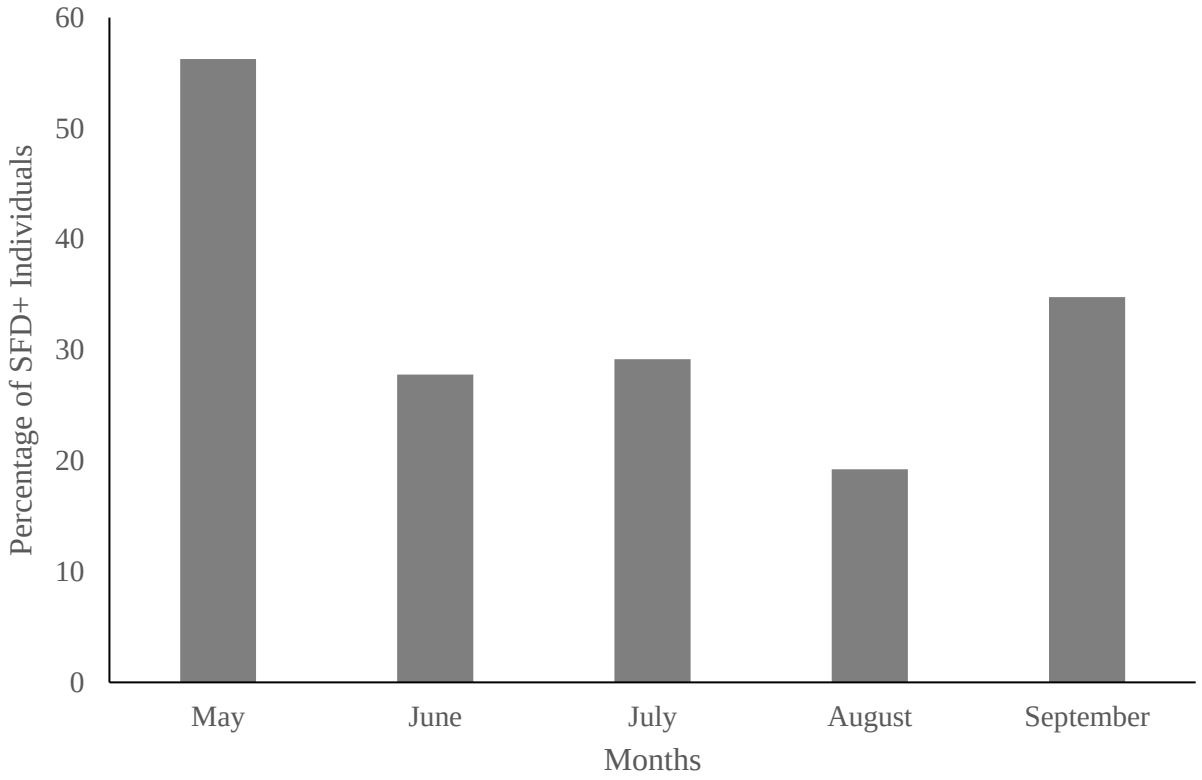

Figure 2-2: Proportion of timber rattlesnakes (*Crotalus horridus*) that tested positive for snake fungal disease each month on the Land Between the Lakes National Recreation Area, Trigg and Lyon County, Kentucky, USA between May and September from 2018-2020.

Table 2-1. The beta (β) coefficients, standard errors (SE), t-values, degrees of freedom (d.f.), P-values, and 95% confidence intervals (CIs) associated with generalized linear models used to assess the influence of mass, length, sex, and time to first blood (baseline only) on baseline and elevated corticosterone levels (ng/mL) in timber rattlesnakes (*Crotalus horridus*) located on Land Between the Lakes in Trigg and Lyon County, Kentucky, USA.

CORT measure	β	SE	t-value	d.f.	P-value	95% CI	
Baseline							
Mass	0.0001	0.0001	0.38	81	0.7	(0.00024	0.000348)
Length	0.0056	0.0052	1.06	81	0.28	(0.00482	0.015961)
Sex	0.4201	0.2671	1.57	81	0.11	(0.11146	0.951626)
Time	0.0008	0.0016	0.49	81	0.62	(0.00241	0.00401)
Elevated							
Mass	-0.0001	0.0001	-0.92	81	0.35	(0.00037	0.000133)
Length	-0.0017	0.0045	-0.38	81	0.7	(0.01068	0.007243)
Sex	0.0579	0.2324	0.24	81	0.8	(0.40444	0.520371)
MagΔ							
Mass	-0.0002	0.0001	-1.5	81	0.13	(-0.0004	5.613233)
Length	-0.0073	0.0041	-1.78	81	0.07	(0.01545	0.000842)
Sex	-0.3626	0.2114	-1.71	81	0	(0.78322	0.057965)

Table 2-2. The beta (β) coefficients, standard errors (SE), t-value, degrees of freedom (d.f.), P-value, and 95% confidence intervals (CI) associated with generalized linear models used to assess relationships between snake fungal disease state and elevated and baseline corticosterone levels (CORT; ng/mL) and CORT reactivity in timber rattlesnakes (*Crotalus horridus*) located on Land Between the Lakes in Trigg and Lyon County, Kentucky, USA.

CORT Status	β	SE	t-value	d.f.	P-value	95% CI	
Baseline							
May	-0.121	0.5149	-0.23	12	0.81	(1.24287	1.00095)
June	-0.4599	0.4948	-0.92	12	0.37	(1.53808	0.61823)
July	0.1025	0.3975	0.25	15	0.8	(0.74476	0.9498)
August	1.0499	0.5744	1.82	17	0.08	(0.16208	2.26186)
September	0.467	0.2942	1.58	17	0.13	(0.15368	1.08765)
Elevated							
May	-0.0537	0.3913	-0.13	12	0.89	(0.90642	0.79884)
June	-0.5044	0.3748	-1.34	12	0.2	(1.32104	0.3122)
July	0.2094	0.341	0.61	15	0.54	(0.51751	0.93626)
August	0.7952	0.347	2.29	17	0.03	(0.06321	1.52723)
September	0.4096	0.3337	1.22	17	0.23	(-0.2944	1.11355)
Reactivity							
May	0.0678	0.3138	0.21	12	0.83	(0.61595	0.75169)
June	-0.046	0.3441	-0.13	12	0.89	(0.79592	0.70378)
July	0.1074	0.2965	0.36	15	0.72	(0.52463	0.73937)
August	-0.2545	0.531	-0.47	17	0.63	(1.37486	0.86583)
September	-0.0561	0.3325	-0.16	17	0.86	(0.75788	0.64549)

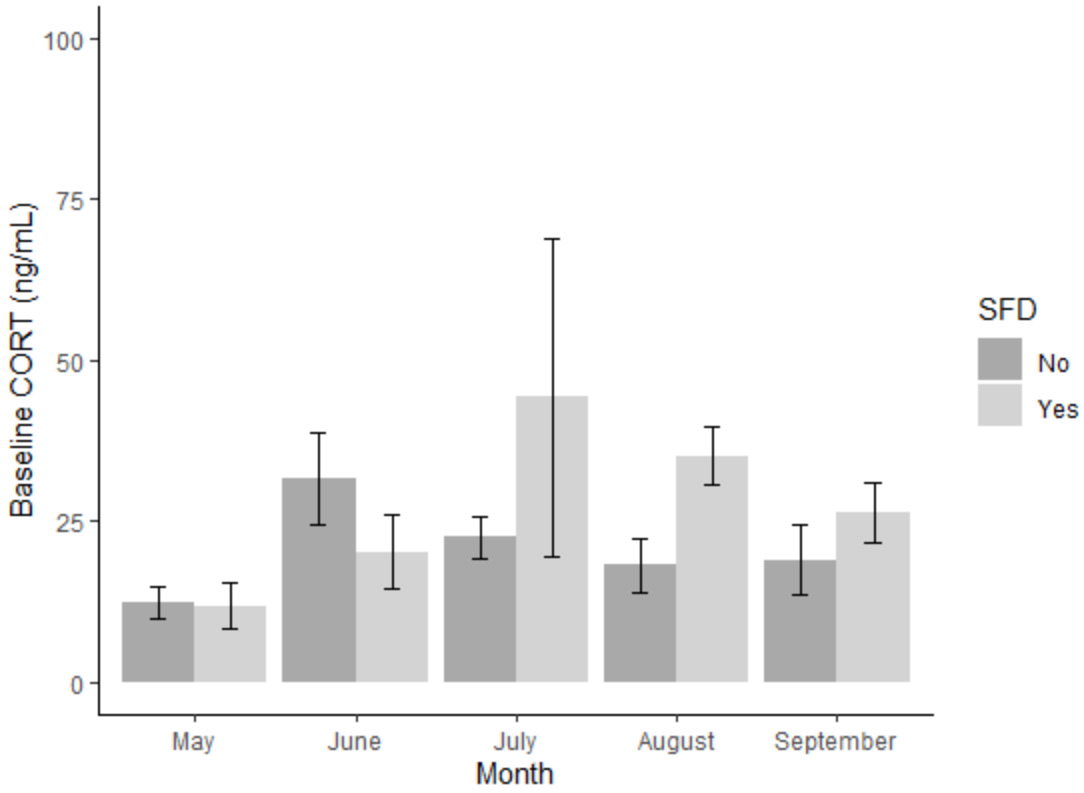

Figure 2-3: Baseline corticosterone (CORT) concentrations (ng/mL) in timber rattlesnakes (*Crotalus horridus)* testing positive (yes) and negative (no) for snake fungal disease (SFD) from May to September 2018 - 2020 in Timber rattlesnakes on the Land Between the Lakes National Recreation Area, Trigg and Lyon County, Kentucky, USA.

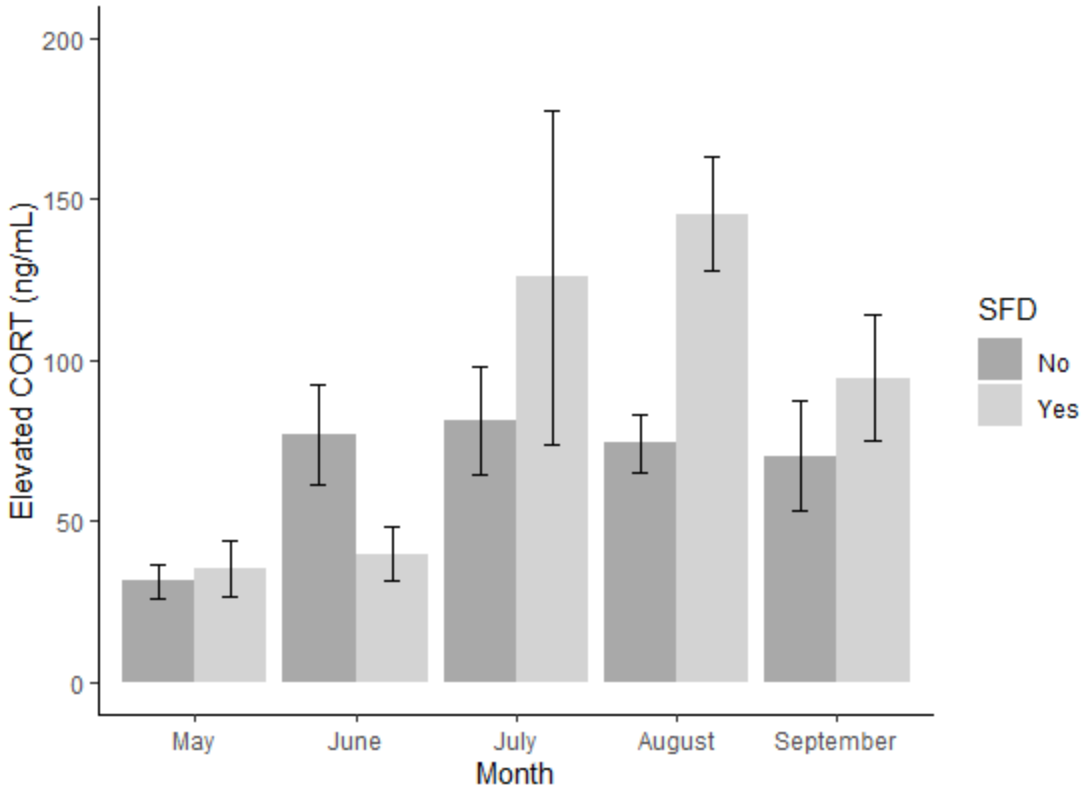

Figure 2-4: Elevated corticosterone (CORT) concentrations (ng/mL) in timber rattlesnakes (*Crotalus horridus)* testing positive (yes) and negative (no) for snake fungal disease (SFD) from May to September 2018 - 2020 in Timber rattlesnakes on the Land Between the Lakes National Recreation Area, Trigg and Lyon County, Kentucky, USA.

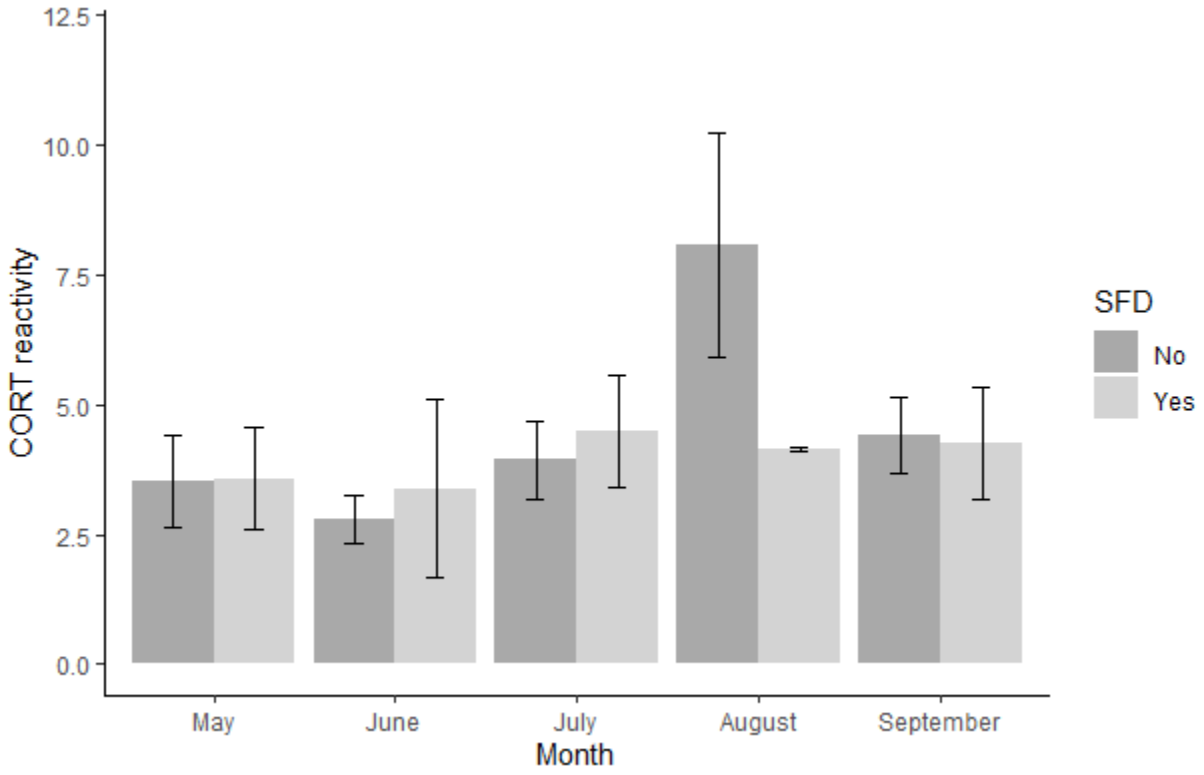

Figure 2-5: Corticosterone (CORT) reactivity (percent increase from baseline to elevated CORT) in SFD positive (Yes) and SFD negative (No) timber rattlesnakes (*Crotalus horridus*) from May to September 2018 - 2020 on the Land Between the Lakes National Recreation Area, Trigg and Lyon County, Kentucky, USA.

Table 2-3. The beta (β) coefficients, standard errors (SE), t-values, degrees of freedom (d.f.), P-values, and 95% confidence intervals (CI) associated with associated with generalized linear models used to assess relationships between the proportion of times a timber rattlesnake (*Crotalus horridus*) tested positive for snake fungal disease (SFD positive rate) and mean baseline and elevated corticosterone (CORT; ng/mL), mean CORT reactivity, and the coefficients of variation for baseline and elevated CORT and CORT reactivity on Land Between the Lakes in Trigg and Lyon County, Kentucky, USA.

CORT Measurement	β	SE	t-value	d.f.	P-value	95% CI	
Mean Baseline CORT	0.1273	0.1465	0.87	9	0.41	(-0.20409	0.4586)
Mean Elevated CORT	0.2168	0.3993	0.54	9	0.6	(-0.68639	1.11997)
Mean CORT reactivity	-0.0006	0.0188	-0.03	9	0.98	(-0.04302	0.04189)
CV base CORT	0.2411	0.3441	0.7	9	0.5	(-0.53733	1.01949)
CV elevated CORT	-0.0928	0.2902	-0.32	9	0.76	(-0.74925	0.56366)
CV CORT reactivity	0.504	0.1791	2.81	9	0.02	(0.09873	0.90926)

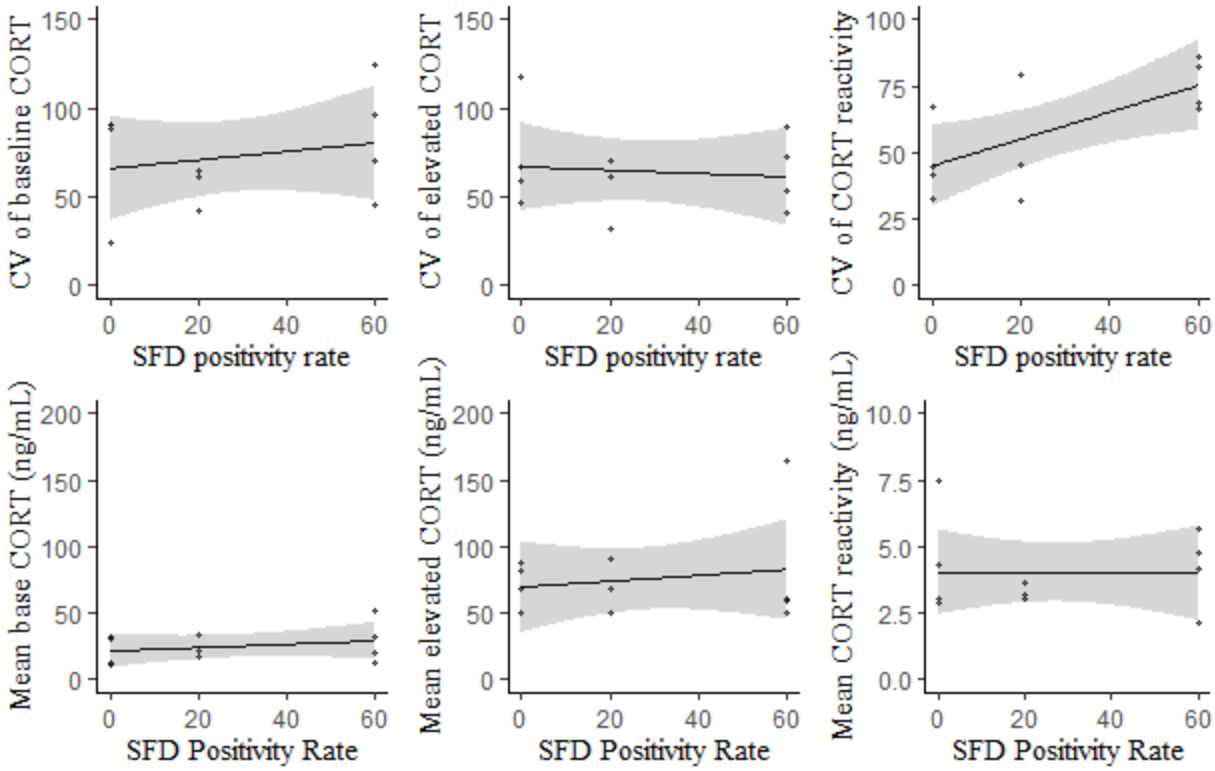

Figure 2-6: Coefficients of variation and averages of baseline corticosterone (CORT;ng/mL), elevated CORT (ng/mL), and CORT reactivity relative to the proportion of snakes that tested positive for snake fungal disease from May to September in 2018 - 2020 in Timber rattlesnakes (*Crotalus horridus*) on the Land Between the Lakes National Recreation Area, Trigg and Lyon County, Kentucky, USA.

Discussion

Contrary to my hypothesis, baseline and elevated CORT and CORT reactivity did not vary relative to disease state across most months. Both baseline and elevated CORT were lowest in May following emergence from hibernacula, with concentrations increasing throughout the summer though no difference was found until August when elevated CORT was 2 times greater in SFD positive snakes as compared with those that were SFD negative. Moreover, individuals that tested positive more often for SFD had greater variability in their CORT reactivity.

One explanation for the greater levels of elevated CORT we observed in SFD positive individuals in August may involve preparation for hibernation. Males travel extensively throughout the breeding season searching for females and expend a large number of calories during this process, which may increase glucocorticoid concentrations (Tomiyama et al., 2010, 2011). At the end of the mating season, males return to their dens and feeding increases concurrently into the fall in preparation for three to five months of hibernation. If an individual is under additional stress from disease during this period, one may expect to see individuals that are SFD positive having a greater response to a stressor, such as handling. Studies are mixed regarding the influence of CORT on reproduction, some suggest elevated levels of CORT can inhibit breeding (Manzo et al., 1994) while others indicate increased CORT levels during breeding may associate positively with reproduction as part of an energy mobilization strategy where glucocorticoids are highest during the most energetically costly time of year, especially in males that may need to travel or defend territories (Romero, 2002). Chronically elevated levels of CORT can lead to reduced survival through immunosuppression and increased risk of infection as has been observed in lizard species (Tylan et al., 2020); however, it is important to note that overall, I did not find large differences between SFD positive and SFD negative snakes

in regard to CORT. Research on Pygmy rattlesnakes has shown that greater baseline CORT levels are associated with individuals that have severe SFD as opposed to moderately infected or uninfected individuals (Lind et al., 2018). One factor that may account for a lack of difference in baseline CORT in my study may be the lack of severely infected individuals. Aside from CORT variability, perhaps SFD in Timber rattlesnakes does not have a large effect on baseline CORT, elevated CORT and CORT reactivity which lends support for taxa specific studies with possible population genetics components.

The stress response is an adaptive mechanism for the maintenance of homeostasis (Anisman and Merali, 1999) and should be consistent when stressors are encountered. Though most work in humans has focused on psychological disorders rather than infectious disease, evidence from humans suggests exposure to repeated stressors can result in dysregulation of the HPA-axis (Makhathini et al. 2017). Thus, HPA-axis dysregulation in response to repeated infections with SFD could account for the greater variability in stress reactivity I observed. Similar to our study, viral infections in mice resulted in HPA-axis dysregulation (Dunn et al., 1989; Silverman et al., 2005). Like viruses, fungal infections are known to elicit a robust immune response (Blanco and Garcia 2008). One explanation for why apparent HPA-axis dysregulation and a more variable stress response occurred in timber rattlesnakes that tested positive more often may be related to the physiological stress and associated HPA-immune interactions of *Ophidiomyces* infection. Though the stress response is adapted by evolution to offer short term benefit in restoring homeostasis, long term or chronic activation may be maladaptive and lead to longer-term implications for an organism’s fitness (Girod and Brotman, 2004).

Individuals transitioned in and out of disease state within and between active seasons. PCR-positive *Oo* varies from month to month in certain individuals as does the presence of clinically suspect lesions. Anecdotally, it appeared that individuals that tested positive went through ecdysis more often over the course of a season. Research has suggested ecdysis may be a strategy for shedding infected skin in individuals with confirmed clinical disease (Lorch et al., 2015). In addition to monitoring monthly plasma glucocorticoids, it may be of value to document incidences of ecdysis as well as monitoring the regulation or secretion of ecdysis triggering hormone (ETH) in individuals who appear to be free of clinical disease and those with apparent SFD.

Some studies indicate a high mortality rate associated with SFD (Allender et al., 2011; Clark et al., 2011) while others indicate SFD associated mortality is not a significant source of mortality for wild snakes (Davy et al., 2021; McKenzie et al., 2021). Overall mortality in this population was low (no more than 20%) which corresponds with existing data on SFD mortality in otherwise healthy populations (McKenzie et al. 2021). The Land Between the Lakes National Recreation Area allows for approximately 170,000 acres of contiguous gene flow between subpopulations of snakes, which may prevent a loss of heterozygosity and associated immunological dysfunction (Reid et al., 2003). Management consists of selective logging and prescribed fire but overall, the site has little anthropogenic stress; however, the lack of continuous gene flow, in areas with differing conditions (e.g. relatively humidity, xeric versus mesic conditions, higher or lower average temperatures) where human pressure (e.g habitat fragmentation/destruction and urbanization) is more intensive may account for the relatively high percentage of SFD positive individuals found in populations of some species across the eastern United States (Allender et al., 2011; Clark et al., 2011).

CHAPTER 3

SURVEY OF SNAKE FUNGAL DISEASE PRESENCE IN WESTERN KENTUCKY

Synopsis

Dermal lesions have been documented on snakes for decades (Dillberger and Abou-Gabal, 1979; Jacobson, 1980). However, it was not until 2006 when a disease associated with lesions on snakes, Snake Fungal Disease (SFD), was documented and described (Allender et al., 2011; Clark et al., 2011). Specifically, following a particularly rainy season, facial and somatic lesions were documented in a population of timber rattlesnakes in New Hampshire and since then SFD has been documented throughout the eastern United States and Europe. Surveys for SFD are increasing globally and it is important we understand the distribution of SFD in different regions to inform and target our conservation efforts. Snake fungal disease has been documented in Kentucky with a particular focus on the bluegrass region, but little has been done in western Kentucky. Thus, my objective was to document the presence of SFD in western Kentucky, particularly the Jackson Purchase region. Beginning in the spring of 2018 through spring 2021, we sampled snakes from a wide range of taxa across the Land Between the Lakes National Recreation Area and the neighboring 8 counties of the Jackson Purchase region. We used nighttime road cruising and hiking to collect and swab 124 individuals from 18 species of snakes. I used Quantitative Real-time PCR to evaluate the presence of *Oo* on the snakes sampled and documented the presence of lesions consistent with SFD. Samples were taken across local snake taxa with 48% being from crotaline pit vipers and 52% from colubrids. Sixteen percent of snakes tested positive for SFD across 3 years of sampling effort, which was lower than other studies, and underscores the importance of regional survey efforts. The value of conducting disease inventories for management, conservation, and wildlife epidemiology cannot be

understated in a world that is seeing a greater degree of disease emergence associated with wide scale anthropogenic changes to landscapes worldwide.

Background

Fungi are versatile, opportunistic, and wide-ranging pathogens that are difficult to treat and nearly impossible to eradicate (Liu et al., 2018). Some of the most devastating diseases in wildlife are fungal pathogens (Hoyt et al., 2021; Scheele et al., 2019). The fungus, *Batrachochytrium dendrobatidis* (*Bd*) has resulted in the loss of more species (~ 90) than any other known pathogen since 1998 (Scheele et al., 2019) and *Pseudogymnoascus destructans*, the causative agent of White-nose Syndrome (WNS) has resulted in the loss or decline of several bat species in North America (Dzal et al., 2011; Frick et al., 2010; Hoyt et al., 2021). Similarly, snake fungal disease (SFD), caused by the fungus *Ophidiomyces ophiodiicola* (*Oo*), may be driving population declines in squamates (Latney and Wellehan, 2013). Yet, fewer studies have been conducted to understand and quantify the influence of fungal diseases on squamates.

Fungal lesions have been observed on many snake species over time (Dillberger and Abou-Gabal, 1979; Jacobson, 1980; McKenzie and Green, 1976); however, in the case of snake fungal disease (SFD) the high level of mortality in some populations has become a concern. Numerous SFD surveys have been conducted across North America and in Europe (Allender et al., 2016; Franklinos et al., 2017; Guthrie et al., 2016; Hileman et al., 2018; Lorch et al., 2016; Patterson et al., 2021; Snyder et al., 2020). Surveys in Kentucky have been largely restricted to the inner bluegrass region of the commonwealth (McKenzie et al. 2020). The extreme western region of the state, known as the Jackson Purchase, and the Land Between the Lakes National Recreation Area (LBL) have not been thoroughly evaluated in terms of *Oo* presence. The

presence of *Oo* should be examined throughout all ecoregions, as *Oo* presence and likelihood for SFD infection may be influenced by different climate, land cover, and land use patterns. Snake species richness in the western region of Kentucky is greater and thus there are more species that may be influenced by SFD. Therefore, it is important to understand how *Oo* is distributed there. Thus, my objective was to assess the presence of *Oo* and SFD in snakes within the Land Between the Lakes National Recreation Area (LBL) and 8 Jackson Purchase Counties.

Methods

Study Site

I conducted my study within the Jackson Purchase Region of western Kentucky, including the Land Between the Lakes National Recreation Area (LBL) (Figure 3-1, 3-2). The region consists of multiple ecosystem types including upland oak-hickory forest, oak savannah, and old growth bald cypress wetland. Established in 1963, The LBL is a 69,201-ha upland oak-hickory hardwood preserve interspersed with prairie and agriculture designed for recreational activities such as hiking, mountain and dirt biking, fishing, hunting and limited agriculture. The LBL is managed by the U.S. Department of Agriculture Forest Service. The surrounding 8 counties within the Jackson Purchase are characterized by intensive agriculture, oak-hickory forests, and sporadic urban environments with the easternmost counties of Calloway and Marshall bordering the Tennessee River impoundment. All private property utilized for this study was accessed with

permission.

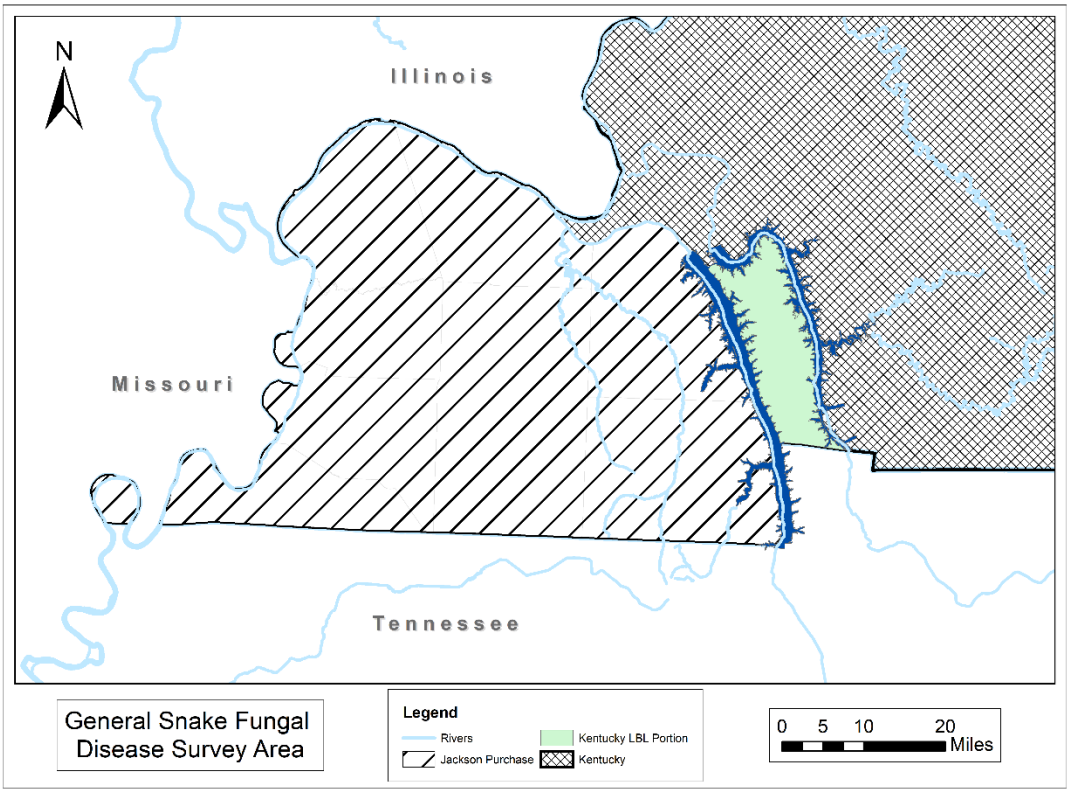

Figure 3-1: The 8-county region of the Jackson Purchase (dashed) and the 69,201 ha Land Between the Lakes National Recreation Area (unshaded) used for the generalized Snake Fungal Disease survey across multiple taxa between May 2018 and April of 2021.

Field Sampling

Beginning in the spring of 2018 we began collecting snakes opportunistically via hiking and road cruising. Sampling typically began in March and went through the end of October. Occasionally, fresh roadkill snakes were also sampled. All collection was conducted with a USDA Forest Service Special Use Permit (#LBL18178) and a Kentucky Department of Fish and Wildlife Resources collection permit (#SC1811061) and approved by the Murray State University IACUC committee.

I swabbed the dorsal, ventral, and facial aspects of each snake collected. For the swabbing procedure I used sterile Rayon applicators dipped into 80 μL of DI water. I passed a single swab along the dorsal, ventral, and facial aspects of the snake five times, rotating the swab slightly each time so that all sides of the swab came into contact with the skin. If I observed a skin lesion, I swabbed the lesion and skin immediately adjacent by passing the swab over the affected skin five times and rotating the swab after each pass as described above. I then placed the swab into a 2 mL collection vial and stored them at -20°C until further processing could be conducted. For a subset of 20 timber rattlesnakes associated with another study I did separate swabs on the dorsal, ventral, and facial aspects and for each lesion and later pooled these samples across the same snake.

DNA Extraction procedure

Prior to DNA extraction, the bench and pipettes were cleaned with DNAoff and rinsed with 100% EtOH. Approximately 100 mg of 0.5 mm zirconium beads were measured and placed into O-ring tubes (with conical bottom) containing the sample swab. Prepman Ultra lysis buffer (125 μl) was added to tubes to cover the beads and swab. Heat block was turned on and set to 100C. Tubes were placed in a disruptor and homogenized for 45 seconds. This was followed by centrifugation for 30 seconds at 13,000 x g to settle all material to the bottom of the tube. Both processes above were repeated again in stepwise fashion and tubes were placed on a heat block at 100C for 10 minutes. Tubes were then removed and allowed to cool for an additional 2 minutes. The tubes were placed back in the centrifuge and spun at 13,000 x g for 3 minutes. A 200 uL pipet and filtered tip were used to extract the supernatant from each sample. The supernatant was combined for all samples from a specific time spot and placed in a separate 1 mL microcentrifuge tube and labeled. Samples were then moved to a -20 C freezer.

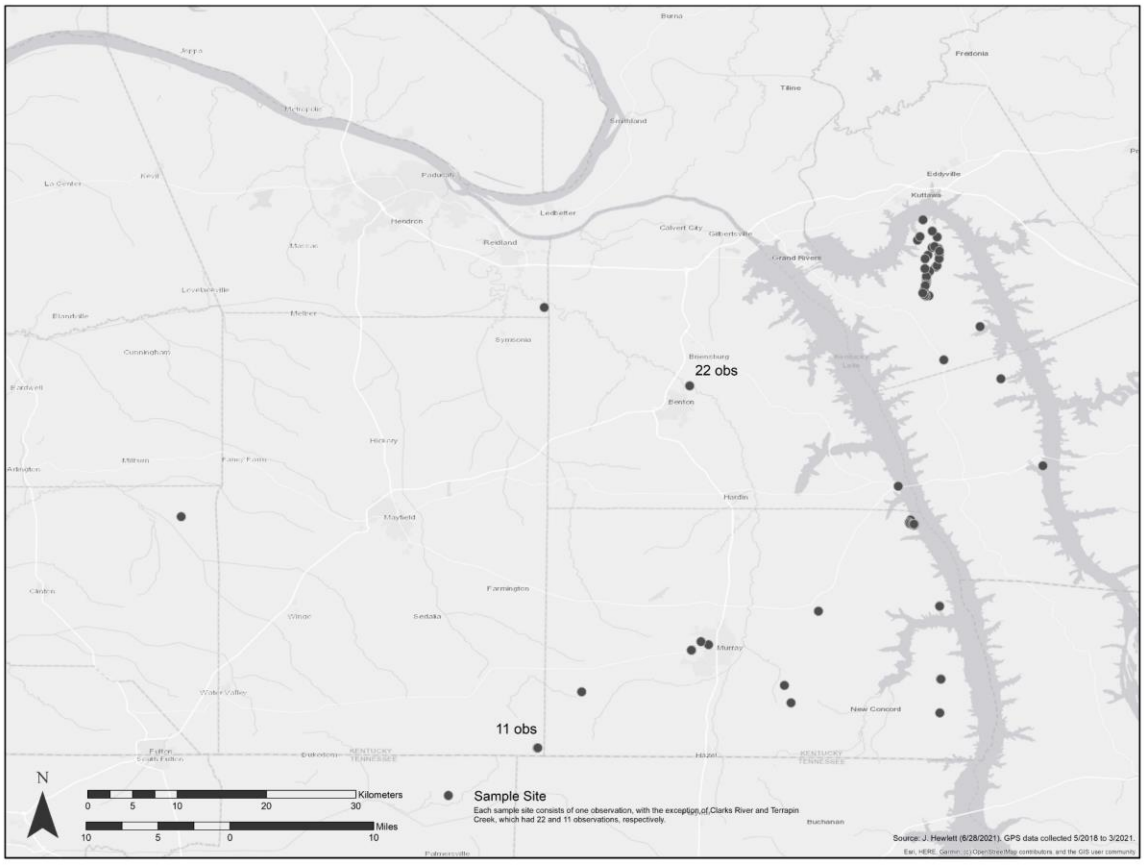

Figure 3-2: Locations of snakes surveyed during the active seasons between May 2018 and April 2021in the 8-county region that makes up the Jackson Purchase and the Land Between the Lakes National Recreation Area. Multiple individuals were found at single points during emergence from hibernacula and those observations are noted.

qPCR

The qPCR protocol I used was developed by Bohuski et al. (2015). I utilized a QuantiFast Probe PCR +ROX Vial Kit along with Bioresearch Technologies targeted forward and reverse primers and probes. The ABI 7500 Fast PCR Machine was used to read reaction well content in the Applied Biosystems® MicroAmp® Fast Optical 96-Well Reaction Plate. I classified snakes as SFD positive if they had lesions and a positive skin swab (Baker et al., 2019).

Analyses

Snakes were categorized as having lesions regardless of SFD status, being PCR-positive for the presence of *Oo*, having no lesions while testing PCR-negative for *Oo* (i.e., clean), no lesions but PCR-positive for *Oo*, lesions present but PCR-negative for *Oo*, and lastly, SFD positive which we defined as having both clinically significant lesions and PCR-positive for *Oo*.

Results

We collected 124 individuals from 18 species of snakes (Table 3-1, Figure 3-3). Forty-eight percent of snakes were crotaline pit vipers (n = 60; 3 species across 3 genera) and 52% were Colubrids (n = 64; 15 species across 12 genera). Most snakes captured were cottonmouths (*Agkistrodon piscivorus*; 27.5%), timber rattlesnakes (*Crotalus horridus*; 20%), and gray rat snakes (*Pantherophis spiloides*; 9.5%). Sixteen percent (n=20) of snakes were SFD positive as they tested positive for the presence of *Oo* and had visible lesions (Figs. 3-4 and 3-5). Twenty-one percent (n=26) of snakes had visible dermal lesions consistent with infection but tested negative for the presence of *Oo*. Twenty-two percent (n=27) tested positive for the presence of *Oo* but had no visible lesions (Figure 3-6.) Fifty-six percent (n=70) of snakes had no external lesions and tested negative for *Oo*. I found that 6.5% (n=8) of snakes had no clinical lesions and tested positive for *Oo*.

Table 3-1: The number and percent of snakes with Oo+, no lesions and Oo- (clean), no lesions and Oo+ , lesions and Oo-, and SFD+ (lesions as well Oo+) that were captured between spring 2018 and spring 2021 as a part of a survey for snake fungal disease completed in western Kentucky, USA.

Species	n	Oo + (%)	No Lesions/ Oo- (%)	No Lesions/Oo+	Lesions/Oo - (%)	SFD + (%)
Agkistrodon piscivorus	34	0 (0)	31 (91)	0 (0)	3 (9)	0 (0)
Carphophis amoenus	1	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)
Coluber constrictor	1	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)
Crotalus horridus	25	18 (72)	5 (20)	4 (16)	2 (8)	14 (56)
Diadophis punctatus	9	0 (0)	1 (11)	0 (0)	8 (89)	0 (0)
Farancia abacura	1	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)
Heterodon platirhinos	2	0 (0)	2 (100)	0 (0)	0 (0)	0 (0)
Lampropeltis calligaster	2	0 (0)	2 (100)	0 (0)	0 (0)	0 (0)
Lampropeltis elapsoides	2	0 (0)	1 (50)	0 (0)	1 (50)	0 (0)
Lampropeltis nigra	7	2 (43)	4 (57)	1 (14)	0 (0)	2 (33)
Lampropeltis triangulum	9	1 (11)	5 (56)	0 (0)	3 (33)	1 (11)
Nerodia erythrogaster	3	2 (67)	0 (0)	0 (0)	1 (33)	2 (67)
Opheodrys aestivus	4	0 (0)	3 (75)	0 (0)	1 (25)	0 (0)
Pantherophis spiloides	12	3 (25)	6 (50)	2 (17)	3 (25)	1 (8)
Sistrurus miliarius	1	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)
Storeria dekayi	1	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)
Thamnophis sirtalis	7	1 (14)	3 (43)	1 (14)	3 (43)	0 (0)
Virginia valeriae	3	0 (0)	3 (100)	0 (0)	0 (0)	0 (0)
	124	22%	56%	6.5%	21%	16%

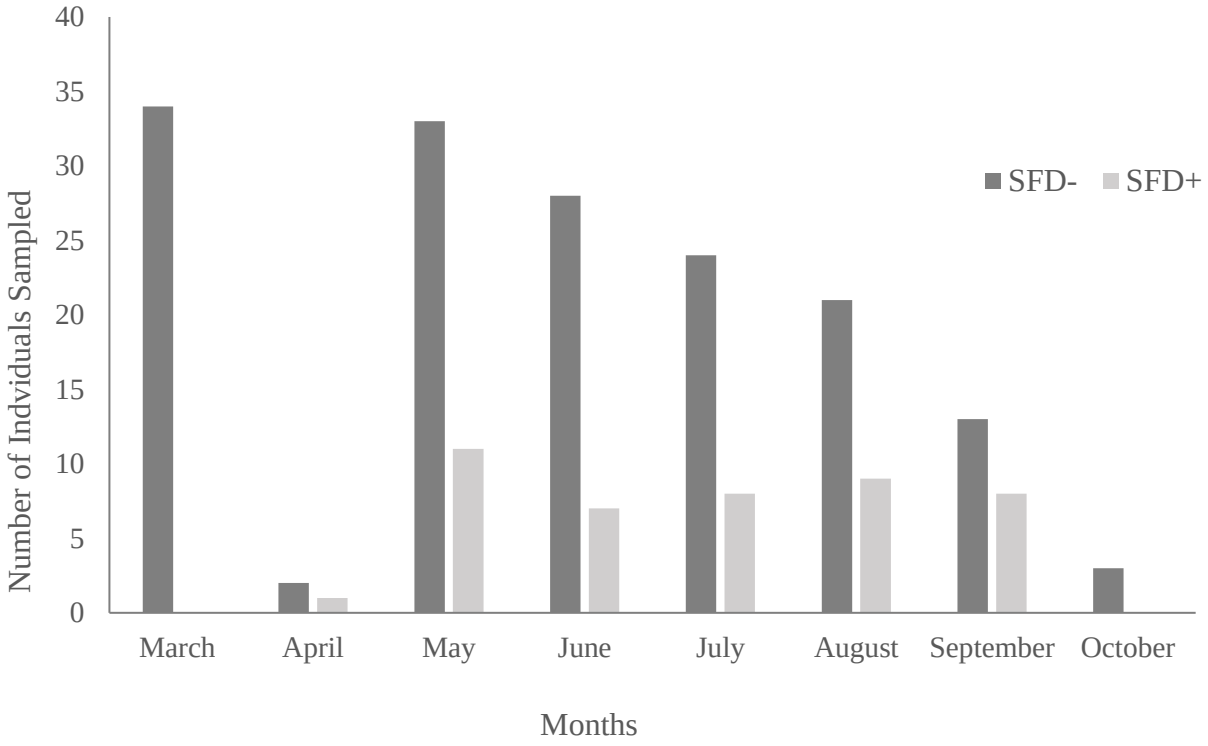

Figure 3-3: The number of individual snakes sampled and snake fungal disease status per month between May 2018 and April 2021 throughout the Jackson Purchase Region and the Land Between the Lakes National Recreation in western Kentucky, USA.

Figure 3-4: A black kingsnake (*Lampropeltis getula nigra)* with clinical signs of SFD fungal disease on the rostral and labial scales of the face. This snake had clinically significant lesions and tested *Ophidiomyces* positive on qPCR.

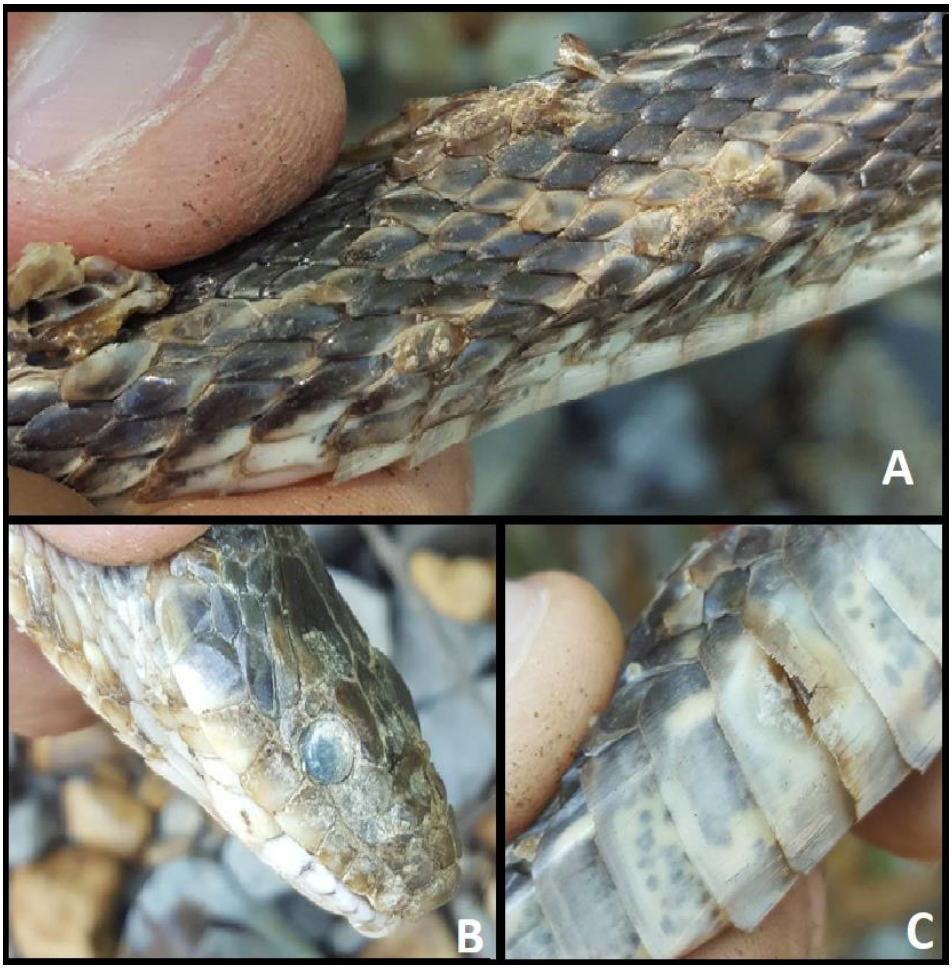

Figure 3-5: A) Dysecdysis: Incomplete shedding and flaking of the skin associated with snake fungal disease. B) Ocular, labial and rostral scales covered in dried yellow plaques. C) Crusts on ventral scales that are commonly associated with infection by *O. ophiodiicola*. The *Pantherophis spiloides* specimen depicted was PCR-positive for snake fungal disease.

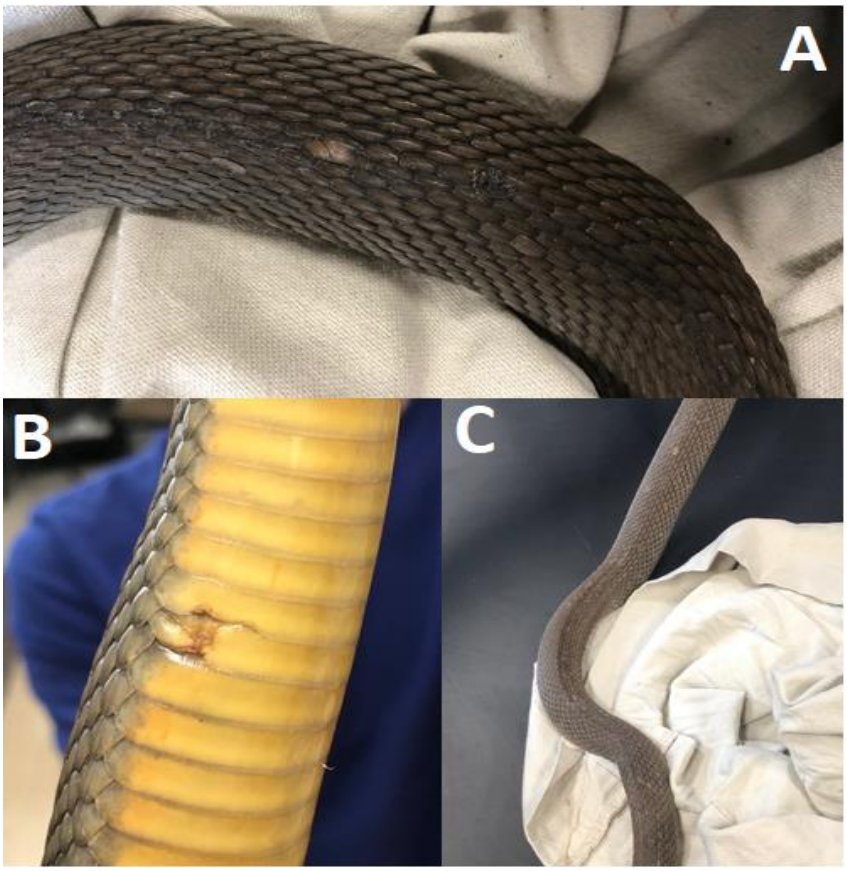

Figure 3-6: A) Swelling and missing scales on the dorsal aspect of a *Nerodia erythrogaster*. B) A gross ventral lesion that is consistent with *Ophidiomyces*. C) Numerous dorsal lesions consistent with clinical Ophidiomycosis. This individual did not test positive for the presence of *O. ophiodiicola* DNA.

Discussion

As with other studies (e.g., Guthrie et al. 2016, McKenzie et al. 2019), most of the lesions I documented were mild in nature with some exceptions (Figures 3-4 and 3-5) and the presence of clinically suspect lesions alone did not always indicate infection by *Oo*. The reliability of clinical lesions alone is generally not diagnostic of SFD as many different fungal pathogens can result in dermal lesions not associated with *Oo* (Dillberger and Abou-Gabal, 1979; Jacobson, 1980; McKenzie and Green, 1976). However, the presence of *Oo* on some snakes without lesions may indicate snakes can carry the fungus and either not become infected with SFD, or be at the early stages of infection, or have an internalized infection. Further research should investigate the potential for snakes to act as carriers and why some snakes are able to survive the disease whereas others (see Figure 3-4) are strongly affected by SFD.

We had approximately 23% fewer detections of SFD positive snakes compared to a similar study conducted, which largely focused sampling in the eastern portion of Kentucky (McKenzie et al., 2019). One reason for the disparity in detections between our studies may involve the proportion of aquatic species sampled by McKenzie et al. (2019) as aquatic species may have greater rates of infection by *Oo* compared with terrestrial species (McKenzie et al. 2019; Lorch et al. 2016). However, I found that terrestrial species tested positive for SFD more frequently than aquatic species. In terms of water snakes (genus *Nerodia*) I found that 67% (n = 2 of 3) of captures tested positive for SFD. However, no Cottonmouths (n = 34), a species that requires similar habitat components as water snakes, tested positive for SFD during the survey. Conversely, a greater proportion of timber rattlesnakes tested positive for SFD. The timber rattlesnake is a species that prefers upland xeric environments and may be more susceptible to fungal infections in environments with shifting moisture contents because of increased rainfall.

This research highlights why studying multiple species across a wide geographic range in variable ecoregions is important to obtain a more complete cross-section of infectivity across populations.

Given increasing human populations are likely to lead to larger landscape level effects that will ultimately influence wildlife disease risk, surveys such as the one I conducted will become increasingly important. A more long-term monitoring plan could be implemented to detect missed cases and I recommend the use of other techniques such as drift fences, placement of artificial cover, and trapping for aquatic species. It is critical determine whether sampling bias plays a role in detection of SFD as well as potential differences in individual susceptibility to infection based on geographic range as well as taxa. In terms of management, conservation, and wildlife epidemiology it would be helpful to fully document the extent of *Oo* presence on the landscape and the potential for greater effects as we experience climate regime shifts caused by climate change.

CHAPTER 4

CONCLUSION

Snake population declines may pose consequences for public health as snakes, acting as predators, may reduce zoonotic disease transmission from prey to humans (Kabay et al., 2013; Keesing et al., 2010; Ostfeld and Holt, 2004). Given this and other important roles snakes play in ecosystems, it is critical we understand the pathobiology and ecology of snake fungal disease (SFD), including possible anthropogenic drivers of the disease in wild populations. These data are vital to inform future management practices related to SFD in snake populations that are in decline or may be at risk of declines with increasing anthropogenic stressors. With these increasing anthropogenic stressors, we must understand how stress affects SFD dynamics. I found that SFD has little influence on snake baseline and or elevated stress or stress reactivity, as measured via corticosterone (CORT). However, I did find a difference in elevated levels of CORT with SFD positive snakes having greater elevated CORT levels in August compared to SFD negative snakes. These greater levels of elevated CORT could influence how snakes respond to a natural stressor, such as a predator, and how they use or move across the landscape which could carry fitness consequences. Alternatively, these greater levels of elevated CORT could increase a snake's survival. In some wildlife species (e.g., *Desmognathus* salamanders) elevated CORT is associated with a decrease in exploratory behavior and thus a greater degree of predator avoidance (Sullivan et al., 2021).

My study was also one of the first to document CORT variability in a wild snake population and to assess differences in CORT variability based on SFD status. Though I found no differences in average baseline CORT, elevated CORT, and CORT reactivity or baseline and elevated CORT variability, I did find a positive correlation between variability in CORT

reactivity and the proportion of times snakes tested positive for SFD (Chapter 2). Ultimately this suggests chronic stressors, either natural or anthropogenic, could result in dysregulation of the HPA-axis and thus come with costs in terms of fitness as a result of an inconsistent response to stress.

I also documented the presence of SFD in several counties within western Kentucky, including the Land Between the Lakes National Recreation Area. We found a relatively low number of snakes with SFD (16%) in the snakes we sampled (Chapter 3) compared to a similar survey in eastern Kentucky. Interestingly, other studies have suggested aquatic snakes should be more prone to infection with SFD, but we did not find that in our survey as the cottonmouth, the aquatic snake we captured in greatest numbers, did not test positive for SFD at all. This underscores the importance of monitoring for SFD in different regions as the disease dynamics may differ. This information will be important in determining the overall extent of *Oo* on the landscape as well the species level implications of the disease. As human encroachment continues to lead to habitat loss, inbreeding depression, direct persecution, and the importation of pathogens becomes more common there is no guarantee that ambient levels of SFDs will remain low into the future.

Though I did not address it in my study, future studies should address the interactive effects of stress physiology, inbreeding depression, loss of heterozygosity in MHC allele loci, along with habitat destruction, fragmentation and altered disturbance patterns on SFD dynamics. Physiology combined with immunogenetics and ecology can provide a greater insight into the current and future state of wildlife diseases.

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